MBMG
Metabarcoding & Metagenomics

Metabarcoding and Metagenomics 10: 321–337 (2026)
DOI: 10.3897/mbmg.10.177451

Emerging Technique

Live and dead fish shed different amounts of intact cells: Implications for advancing environmental DNA methodologies

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Abstract

Environmental DNA (eDNA) analysis has significantly contributed to biodiversity monitoring due to its sampling simplicity and inherent high sensitivity. While the technique has matured considerably for species detection and characterising community compositions via metabarcoding, its applications in population and landscape genetics remain underexplored, primarily because these fields require the extraction of high-quality genetic information from samples. To facilitate further technical development in this domain, we investigated the availability of intact fish cells in water — a source expected to yield high-quality DNA templates — by performing a five-day replicated tank experiment using live and dead Rainbow Trout (*Oncorhynchus mykiss*) as experimental subjects for eDNA production. Propidium monoazide (PMA), a photoreactive intercalating dye which is permeable to disrupted biological membranes and thereby inhibits PCR amplification of DNA, was employed to differentially quantify PMA-resistant eDNA as a proxy for eDNA derived from intact versus compromised cells relative to the total eDNA pool, based on whether fish were living or dead. Our results showed significantly higher concentrations of total eDNA released by dead fish compared to live fish on Day 2 (12.9×) and Day 3 (7.4×). Furthermore, the amount of PMA-resistant eDNA was significantly elevated in the dead fish treatments on Day 2 (29.3×), Day 3 (31.3×) and Day 5 (5.2×). Collectively, these findings indicate that dead fish release significantly greater amount of both total eDNA and PMA-resistant eDNA, which likely represents eDNA from intact cells, into the surrounding environment compared to live fish. Our established methodology for assessing the availability of intact cells in water and the resulting quantitative data provide crucial insight for advancing environmental genetics approaches within the eDNA framework.

Key words: Disrupted cells, eDNA shedding, intact cells, population genetics, propidium monoazide (PMA), single cell

OPEN ACCESS

Academic editor: Chloe Robinson
Received: 20 November 2025
Accepted: 13 March 2026
Published: 2 April 2026

Citation: Yamanaka H, Hirohara T, Hoy MS, Chase DM, Duda JJ, Ostberg CO (2026) Live and dead fish shed different amounts of intact cells: Implications for advancing environmental DNA methodologies. Metabarcoding and Metagenomics 10: e177451. https://doi.org/10.3897/mbmg.10.177451

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Introduction

Environmental DNA (eDNA) analysis has rapidly become a sensitive and efficient tool for ecological monitoring, enabling the detection of aquatic and terrestrial species from traces of genetic material found in water (Thomsen and Willerslev 2015), soil (Dyson et al. 2024) or air (Polling et al. 2024). This non-invasive approach has repeatedly demonstrated higher sensitivity than conventional survey methods, especially for rare, elusive or invasive species (Fediajevaite et al. 2021; Iacaruso et al. 2025). For instance, in Boardman Lake, Michigan, USA, eDNA analysis proved to be more sensitive for detecting fish species relative to traditional survey methods, specifically fyke and gill nets, per sampling event (Gehri et al. 2021).

The capacity for eDNA to yield quantitative data on species relative abundance and diversity, combined with its cost-effectiveness, has resulted in its expanded use in biodiversity and ecosystem assessments (Tillotson et al. 2018; Karlsson et al. 2022). Since the first application of eDNA methods to detect vertebrates in 2008 (Ficetola et al. 2008), there has been a proliferation of methodological refinements, motivated by aims to obtain increasingly high-quality population genetic and genomic information from complex environmental samples (e.g. Yoshitake et al. (2021) for inferring abundance by counting haplotypes; Jensen et al. (2021) for using bait probes to capture genome-wide information). Traditional population genetic studies have typically relied on invasive sampling that requires handling organisms to collect tissue for analysis. However, recent advances in eDNA analysis have made it possible to recover population and even metaphylogeographic insights from environmental samples (Riley et al. 2025; Amin et al. 2026). Furthermore, overcoming the technical barriers to obtaining multilocus data from eDNA would increase the reliability of eDNA-based population genomic inferences (Wilcox and Jensen 2022).

The level of detail achievable in ecological and conservation genetic studies is strongly influenced by the quality of DNA recovered from samples. A primary challenge for eDNA is that it is often highly fragmented (Bylemans et al. 2018; West and Deagle 2025), particularly for extracellular eDNA that is highly prone to degradation by microbial activity in the environment (McKnight et al. 2024). Although suitable for basic eDNA analyses, such degradation can impede the recovery of multiple genetic loci, thereby limiting the power of genomic analyses as confirmed in tissue-derived DNA samples (Campbell and Narum 2009). Provided they can be practically applied, technologies that preferentially recover intracellular DNA would greatly benefit environmental genomics by enabling more robust multilocus genotyping as suggested by Blainey (2013) in microbial studies. Although single-cell genomic approaches have been used in microbial ecology (Weiner et al. 2016; Nishikawa et al. 2022), their application to macro-organismal eDNA has not been widely implemented. While this technique could be transformative by overcoming the loss of physical linkage (a major hurdle in eDNA population genetics, which requires preserving sample-level individuality), its implementation is currently hindered by the uncertainty of intact cell availability in the environment (Couton et al. 2023). This is because single-cell analysis enables the recovery of individual-specific genomic information from bulk eDNA, which is a mixture of genomic material from multiple individuals (Andres et al. 2023). Future integration of single-cell and eDNA technologies,

facilitated by improved separation and isolation of eDNA components, could further strengthen ecological inference, including absolute abundance or the presence of hybrid individuals (Minamoto 2022).

To infer eDNA derived from intact cells as a component of eDNA, propidium monoazide (PMA) was used in this study. PMA is a photoreactive intercalating dye that is permeable to disrupted cell membranes (Nocker et al. 2007). Upon exposure to intense visible light, the photoreactive properties of the dye induce the formation of covalent bonds between PMA and the targeted DNA molecules, which effectively inhibits subsequent PCR amplification (Nocker et al. 2007). Conversely, the DNA in intact membranes remain shielded from the inhibitory effects of PMA, thereby allowing for its selective detection via PCR. We interpret PMA-resistant DNA as a proxy for membrane-protected compartments consistent with intact cells. By applying a PMA-based protocol, we discriminated a PMA-resistant fraction consistent with intact compartments from DNA present in extracellular or membrane-compromised states, though PMA resistance alone does not uniquely identify whole cells; protected non-cellular compartments (e.g. extracellular vesicles) may contribute to the observed signal. To align with the definition of three eDNA states in Kirtane et al. (2023), the adsorbed and dissolved DNA states must be susceptible to PMA. Furthermore, some components of the membrane-bound DNA, the third state defined in Kirtane et al. (2023), may also be susceptible to PMA. This is because the mere physical association of DNA within a cell membrane does not necessarily imply the membrane is fully intact. In summary, PMA-based eDNA analysis provides an estimate of the PMA-resistant eDNA (likely eDNA in intact cells) concentration in water samples.

We used an experimental approach with Rainbow Trout (*Oncorhynchus mykiss*) to determine the proportion of intact cells in water by measuring the amount of eDNA in intracellular (intact) and extracellular and membrane-compromised form using PMA. Herein, we collectively refer to both DNA in extracellular form and that which is derived from cells whose membranes have been damaged as "extracellular DNA". By comparing tanks containing either live fish or carcasses and analysing the temporal dynamics of intact cell release, we demonstrated how physiological status and post-mortem intervals influence the abundance of intact cells. This approach allowed us to distinguish DNA in intact cells suitable for high-quality genomic analysis from extracellular DNA, which is presumably more degraded and of lesser quality.

Materials and methods

Experiment 1: Confirming the PMA inhibitory effect

All Rainbow Trout used for Experiment 1 (and also for Experiment 2 below) were handled under the Institutional Animal Use and Care Committee protocol number 2008-62 at the U.S. Geological Survey (USGS), Western Fisheries Research Center (WFRC). To confirm that PMA could be applied to our Rainbow Trout eDNA experimental study, we applied the PMA treatment to water samples obtained from a stock tank of Rainbow Trout. The temperature-controlled (ca. 9 °C) tank held approximately 278 litres of water (water source for the USGS wet laboratory facility was filtered and UV-treated water from Lake Washington) with 58 Rainbow Trout (average wet weight = 7.15 g). Water was continuously

replaced in a single pass flow-through system at a rate of ca. 500 litres per hour. We collected water samples from the tank outlet by pouring 0.5 litres into nine disposable volumetric cup replicates. One negative control of 0.5-litre ultrapure water in a disposable cup was also collected and treated as a filtration negative control (FNC) to check for unintended contamination during the experimental process after the sampling. We used the same pair of latex gloves when collecting the water samples. The nine water sample replicates were divided into three treatments: 1) unpasteurised-PMA treated (hereafter uPMA); 2) pasteurised-PMA treated (pPMA) and 3) unpasteurised-untreated (UT), with three water sample replicates for each treatment. The purpose of uPMA was to provide an estimate of the intracellular eDNA concentration in unpasteurised water samples. The purpose of pPMA was to provide an estimate of the eDNA concentration after pasteurisation and PMA treatment. Pasteurisation, an incubation procedure performed at a specified temperature over a specific time, was performed to disrupt the integrity of the intact cell membranes in the water samples so that all eDNA in the water sample would become exposed to the extracellular environment and, therefore, susceptible to the PCR-inhibiting effects of PMA. The purpose of UT was to provide an estimate of the total eDNA concentration in the water sample derived from the combination of intra- and extracellular DNA. The FNC was treated in accordance with the UT samples.

The three pPMA samples were each placed into a sterilised glass bottle and incubated in a water bath at 65 °C for 60 min for pasteurisation. After the incubation, all samples (n = 9 + FNC) were placed into disposable plastic bags (DP16-TN1000; Yanagi, Nagoya, Japan) and vacuum filtered using Sterivex filter cartridges (pore size 0.45 µm; Merck, Darmstadt, Germany) connected to an aspirator and both pPMA and uPMA samples were subjected to PMA treatment following the methods of Hirohara et al. (2021). Briefly, 2 millilitres of 50 µM PMA (PMAxx: Biotium, Fremont, California, USA; adjusted to the concentration with ultrapure water) were added to each Sterivex filter cartridge, which were then incubated in darkness at room temperature for 10 min. After incubation, the PMA treated Sterivex filter cartridges were exposed to strong visible light (wavelength: 465–470 nm) for 15 min to activate PMA, which allows it to form a covalent bond with double-stranded DNA.

DNA was extracted from the Sterivex filters following the protocol in Hirohara et al. (2021). Briefly, the PMA solution in uPMA and pPMA treatments and residual water in UT treatments and FNC were removed from each Sterivex filter cartridge by centrifugation. Buffer AL, including proteinase-K (Qiagen, Valencia, California, USA), was then added to each Sterivex filter cartridge followed by incubation at 56 °C for 20 min on an ACR-100 motorised mini rotator (As One, Osaka, Japan) at 20 rpm. Next, purification of DNA was carried out using DNeasy Blood & Tissue Kit (Qiagen, Valencia, California, USA) following manufacturer's recommendations with a final elution volume of 100 µl. The DNA extractions were stored at -20 °C until downstream PCR experiments.

Real-time PCR was performed on a ViiA 7 Real-Time PCR System (Applied Biosystems, Foster City, California, USA) using a species-specific assay for Rainbow Trout (Wilcox et al. 2015) to measure the concentration of target eDNA in the samples. All samples and FNC were tested together with a non-template control (NTC) with three technical replicates. PCRs were performed in 12 µl reaction volumes consisting of 2 µl of DNA template, 6 µl of Gene Expression Master Mix (Thermo,

Carlsbad, California, USA) and a final concentration of forward and reverse primers at 450 nM each and probe at 125 nM. For the NTC, 2 µl of ultrapure water was added in place of DNA template. All PCR runs included the NTC and DNA quantity standards prepared as a dilution series of synthesised target DNA fragments (gBlocks, Integrated DNA Technologies Inc., Coralville, Iowa, USA). The standards were run in triplicate as an eight-point serial dilution at concentrations of 1×10^1–10^8 copies per reaction. DNA concentrations were estimated as DNA copies per litre (l). Note that we confirmed no sign of PCR inhibition due to the PMA treatment itself by comparing Ct values of spiked internal positive controls between PMA-treated and untreated eDNA samples derived from the same test water.

Experiment 2: eDNA concentration and intracellular/extracellular ratios in tanks containing either live or dead fish

We obtained fish from a stock population used by an unrelated study that had ended shortly before the current work. The experiment consisted of 13 flow-through tanks, each containing 27.5 litres of processed water (see Experiment 1). Each tank received a continuous, single pass water flow at 152 l hr^{-1} maintained at ca. 12 °C and all tanks were aerated over the entire experimental period. Prior to introducing fish, all tanks and accessories were treated overnight with a 10% bleach solution (i.e. 10 ml household bleach:90 ml distilled water) and rinsed with the USGS facility source water.

On day 0 of the experiment, 0.5 litres of tank water was sampled from the surface of each tank using disposable plastic cups to establish that tanks, accessories and water were free of Rainbow Trout DNA prior to fish introduction into the tanks. All fish were anaesthetised in a bath of buffered neutral Tricaine methanesulfonate (MS-222) and their length and wet weight measured before they were introduced into tanks (average wet weight = 26.0 ± 5.9 g, average fork length = 126 ± 11 mm; see Suppl. material 1: table S1 for details). Six tanks were assigned to the live fish block, each tank holding five live fish (hereafter Live Fish block). Live fish were fed bloodworms daily. Six other tanks were assigned to the carcass block, with each tank containing five dead fish (hereafter Carcass block). Prior to their introduction into experimental carcass tanks, fish were euthanised with an overdose of MS-222. One tank served as a negative control tank (no live or dead fish) and was used as a filtration negative control (FNC) to monitor the possibility of unintended cross contamination amongst the samples during the downstream experimental procedures.

Duplicate 0.5-litre water samples were collected from the surface of each tank in both blocks on days 1, 2, 3 and 5. One of the duplicates from each tank was treated with PMA (hereafter PMA-Treated) and the other duplicate was not treated with PMA (hereafter PMA-Untreated). PMA-Treated provided estimates of the PMA-resistant (likely intracellular) eDNA concentration in water samples. PMA-Untreated provided an estimate of the total eDNA concentration in water samples derived from the combination of intra- and extracellular DNA. A 0.5-litre water sample was collected from the negative control tank on days 1, 2, 3 and 5. For each tank, new latex gloves were used to collect water samples. The filtration, PMA treatment, DNA extraction and PCR procedure were conducted in the same manner as Experiment 1. FNC samples were not treated with PMA and were analysed along with the other samples.

Data analysis

We expected that pasteurisation in Experiment 1 would compromise the integrity of intact cell membranes, exposing all DNA molecules to the extracellular environment and, hence, the PCR suppressing effects of PMA. Consequently, the eDNA concentrations measured in the pPMA treatment should, ideally, be zero. However, we found that the proportion of DNA molecules quantified in pPMA was estimated to be 0.394. This suggests that a subset of DNA was either not suppressed by PMA — likely due to insufficient membrane disruption during pasteurisation — or was exposed, but not effectively suppressed by PMA (see Results and also Discussion). Given this imperfect PCR inhibition efficiency of PMA, the eDNA concentrations in PMA-Treated samples in Experiment 2 were calibrated multiplying by a factor of 0.606 (i.e. 1–0.394) to avoid overestimating the amount of PMA-resistant eDNA. These calibrated data were used for data analysis.

To estimate the efficiency of PMA on binding to Rainbow Trout eDNA and suppressing PCR amplification, the eDNA concentrations from Experiment 1 were $\log_{10}$-transformed and analysed by ANOVA and post hoc Tukey's test to estimate statistically significant differences in mean concentration amongst pPMA, uPMA and UT.

The dataset obtained in Experiment 2 was modelled using a generalised linear mixed model (GLMM):

$$\log_{10}(\mathbf{eDNAconcentration}) = \mathbf{DL^{*}PMA\ *\ Day} + (1 \mid \mathbf{Tank})$$

where Log_{10}(eDNA concentration) is the common logarithm of the DNA concentration in 1 litre of tank water, DL is a categorical factor indicating the status of a fish as live or carcass, PMA is a categorical factor indicating PMA treated or not treated, Day is a categorical factor indicating the number of days since the initiation of the tank experiment on Day 0 and Tank was a within-subject random factor. One observation from Tank 7 (Carcass block) exhibited a disproportionately high Cook's distance (> 0.5) and a substantial Pearson residual (> 8), indicating that this data point was a significant outlier that deviated from the overall distribution. Although no physical abnormalities were confirmed within the tank, the extreme value was judged as abnormal and attributed to stochastic technical errors, such as the accidental trapping of a large tissue aggregate or localised contamination. To ensure the robustness of our statistical inferences and prevent these influential outliers from distorting the general trends, all data from this specific tank were excluded from the primary analysis. Note that complete dataset including Tank 7 remains available in Ostberg et al. (2025).

To analyse whether tanks containing living fish and carcasses differed in terms of intracellular and extracellular states, the ratio of PMA-Treated to PMA-Untreated (hereafter, PMA:non-PMA ratio) concentrations were modelled by GLMM:

$$\begin{aligned}&\log_{10}(\mathbf{eDNA\ concentration\ in\ PMA\text{-}Treated})\\ &/\log_{10}(\mathbf{eDNA\ concentration\ in\ PMA\text{-}Untreated})\\ &\quad = \mathbf{DL^{*}Day} + (1 \mid \mathbf{Tank})\end{aligned}$$

where Log_{10}(eDNA concentration in PMA-Treated) and Log_{10}(eDNA concentration in PMA-Untreated) are the common logarithm of the DNA concentration

in 1 litre of tank water and DL, Day and Tank are as indicated in the previous GLMM model. Tank was incorporated as a random factor.

All statistical tests were conducted using R version 4.4.2 (R Core Team 2024). GLMMs were conducted using the lmer function in the R package "lme4" and the output summarisation and multiple comparisons were conducted by joint_tests and emmeans functions in the R package "emmeans". All significance testing on results were done using alpha = 0.05, but, for the multiple comparisons, p-values were adjusted with the Bonferroni method.

Results

Experiment 1: Confirmation of PMA efficacy

The mean eDNA concentrations observed in three treatments were 12,630 ± 3490, 4971 ± 1888 and 522,822 ± 90,462 copies/l for uPMA (unpasteurised-PMA treated), pPMA (pasteurised-PMA treated) and UT (unpasteurised-untreated), respectively. These means were significantly different in estimated mean eDNA concentration amongst the three treatments ($p < 0.001$; Table 1a) and pair-wise comparisons revealed a significant reduction in eDNA concentration in the pPMA treatment compared to UT treatment and uPMA treatments (Tukey's test, $p < 0.001$ and $p = 0.019$, respectively; Table 1b). The mean eDNA concentration in the uPMA and pPMA treatments were 2.42% and 0.951% of the UT concentration, respectively. The mean eDNA concentration in the pPMA treatment represented 39.4% of uPMA, suggesting imperfect suppression of DNA. Given this, 60.6% (i.e. 100–39.4) of eDNA measured in the uPMA treatment was interpreted as the portion of PMA-resistant eDNA that likely existed in intact cells. Based on this result, the eDNA concentrations estimated in the PMA-treatment in Experiment 2 were calibrated to 60.6% of the measured concentration to estimate the amount of PMA-resistant eDNA as a conservative proxy for eDNA in intact cells to avoid overestimation. PCR efficiency and R^2 were 89.185% and 0.996, respectively, for the samples in this experiment.

Experiment 2: The ratio of extra-cellular to intra-cellular eDNA concentration in tanks containing either live fish or carcasses

The estimated eDNA concentration observed in both Live Fish and Carcass blocks fluctuated over time, but especially in the Carcass block (Fig. 1). The GLMM results suggest that DL, PMA and Day had significant effects on eDNA concentration (all $p < 0.001$, Table 2a). Moreover, interaction effects of DL:PMA and DL:Day were also significant ($p = 0.010$ and $p < 0.001$, respectively). This suggests that eDNA from Live Fish and Carcass blocks were differentially affected by PMA and an interaction plot confirmed a slightly stronger suppressing effect of PMA in the Live Fish block (Suppl. material 1: fig. S1).

The effect of DL changed over time with relatively weaker effects on Day1 and Day5 (Suppl. material 1: fig. S2). Multiple comparison testing indicated the mean eDNA concentration was different between Live Fish and Carcass blocks in the PMA-Untreated on Day2 ($p < 0.001$) and Day3 ($p < 0.001$), but not on Day1 ($p = 1$) and Day5 ($p = 0.8186$; Table 2b). In the PMA-Treated controls, eDNA concentration was different between Live Fish and Carcass blocks on Day2 ($p < 0.001$), Day3 ($p <$

Table 1. (a) Analysis of Variance (ANOVA) result for the effect of treatments (pasteurised-PMA treated, unpasteurised-PMA treated, unpasteurised-untreated) on the concentration of Rainbow Trout eDNA in Experiment 1; **(b)** Post-hoc pairwise comparisons to test for significantly different means between treatments. PMA stands for propidium monoazide.

(a)	Df	Sum Sq	Mean Sq	F value	Pr (>F)
Treatments	2	6.963	3.481	208.3	2.86E-06
Residuals	6	0.1	0.017		
(b)		**diff**	**lower 95% CI**	**upper 95% CI**	**P adjusted**
unpasteurised-untreated vs pasteurised-PMA treated		2.037566	1.71368	2.3614518	0.0000028
unpasteurised-PMA treated vs pasteurised-PMA treated		0.412535	0.088648	0.7364205	0.0185522
unpasteurised-PMA treated vs unpasteurised-untreated		-1.62503	-1.94892	-1.3011452	0.0000113

Tests performed on log10-transformed data.

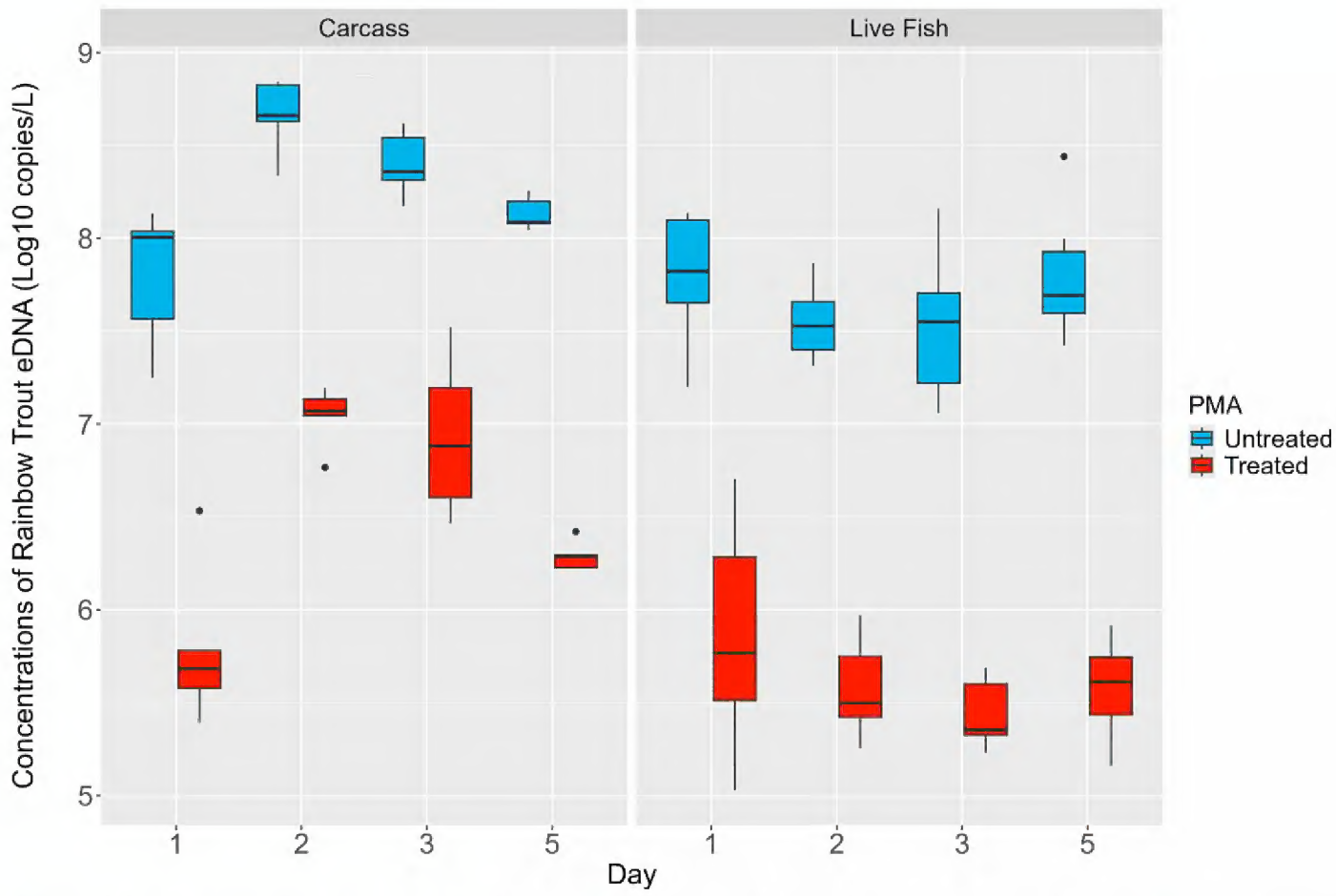

Figure 1. Concentrations of Rainbow Trout eDNA from four treatments in Experiment 2: PMA -Treated and PMA-Untreated for each block of Carcass and Live Fish. Day indicates the days that samples were collected after starting the experiment. Each boxplot is based on six tank replicates for Live Fish and five for Carcass. Within each boxplot, the solid central line represents the median. The lower and upper boundaries of the box indicate the 25th and 75th percentiles (the interquartile range: IQR), respectively. The upper and lower whiskers extend from the box to the highest and lowest values that are within 1.5 * IQR of the hinge. PMA stands for propidium monoazide.

0.001) and Day5 (p = 0.006), but not on Day1 (p = 1; Table 2b). The Carcass block always had higher eDNA concentrations in all cases where significant differences were observed. However, the average magnitude of eDNA concentration compared to the respective Live Fish block was larger when PMA treatment was applied. The magnitudes were 29.3, 31.4 and 5.17 on Day2, Day3 and Day5, respectively (Table 2b; shown as ratio). In the absence of PMA, the magnitudes were smaller and were 12.9 and 7.42 on Day2 and Day3, respectively (Table 2b).

Table 2. Result of generalised linear mixed model (GLMM) analysis on the eDNA concentration data in Experiment 2. **(a)** Significance of each term in the model and interactions; **(b)** Output of multiple comparison tests focusing on Carcass/Live Fish for each Day and use/non-use of PMA. PMA and DL stand for propidium monoazide and dead or live, respectively.

(a)	Model term	df1	df2	F.ratio	p.value				
	DL	1	9	40.313	0.000132993				
	PMA	1	63	975.361	4.76389E-40				
	Day	3	63	7.768	0.000171349				
	DL:PMA	1	63	7.145	0.009559057				
	DL:Day	3	63	25.767	5.36502E-11				
	PMA:Day	3	63	1.089	0.3603282				
	DL:PMA:Day	3	63	1.415	0.2466883				
(b)	**PMA**	**Day**	**contrast**	**ratio**	**SE**	**df**	**null**	**t.ratio**	**p.adjusted**
	Untreated	1	Carcass/Live Fish	1.008	0.461	49.9	1	0.018	1
	Untreated	2	Carcass/Live Fish	12.878	5.886	49.9	1	5.592	<.0001
	Untreated	3	Carcass/Live Fish	7.415	3.389	49.9	1	4.384	<.0001
	Untreated	5	Carcass/Live Fish	2.14	0.978	49.9	1	1.664	0.8186
	Treated	1	Carcass/Live Fish	0.861	0.393	49.9	1	-0.328	1
	Treated	2	Carcass/Live Fish	29.301	13.391	49.9	1	7.391	<.0001
	Treated	3	Carcass/Live Fish	31.378	14.341	49.9	1	7.540	<.0001
	Treated	5	Carcass/Live Fish	5.167	2.362	49.9	1	3.594	0.006

Degrees-of-freedom method: Kenward-Roger.
P value adjustment: Bonferroni method for 8 tests in the section b.
Tests are performed on the log10 scale.
Ratios are shown based on the comparison in linear scale.

Comparing the differences in eDNA shed from live fish and carcass during the five-day tank experiment illustrated that larger amounts of eDNA, for both intracellular eDNA and total eDNA, was shed in carcass tanks. The total eDNA concentration was 5.9 times higher (average during five-day experiment) in tanks with carcasses compared to those with live fish. As for intracellular eDNA, the concentration in the Carcass block was 16.7 times higher, on average, than in the Live Fish block. The proportion of total eDNA represented by intracellular eDNA in Carcass and Live Fish blocks was 4.6% and 1.7%, respectively, when averaged over the five-day experiment.

The GLMM analysis comparing PMA/non-PMA ratios between Live Fish and Carcass blocks revealed significant effects of Day ($p = 0.042$), DL ($p < 0.001$) and their interaction (Day:DL, $p = 0.004$; Table 3a; Fig. 2). The significant effect of the interaction term suggests that the effect of fish status changed over the duration of the experiment. Multiple comparison testing suggested significantly higher PMA/non-PMA ratio in carcass than live fish on Day2, Day3 and Day5 ($p = 0.0029$, $p < 0.001$, $p = 0.0218$, respectively), but not on Day1 ($p = 1$; Table 3b). In the case of Carcass blocks, the ratio differed significantly between Day1 and Day2 and between Day1 and Day3, while in Live Fish blocks, no difference between days was observed (Table 3c). PCR efficiency and R^2 were 90.851–92.071% and 0.997–0.998, respectively, for the samples in this experiment.

In Experiment 2, one FNC (water sample from a control tank without fish) on Day3 contained a small number of Rainbow Trout DNA copies suggesting contamination during water sampling or downstream sample processing. However,

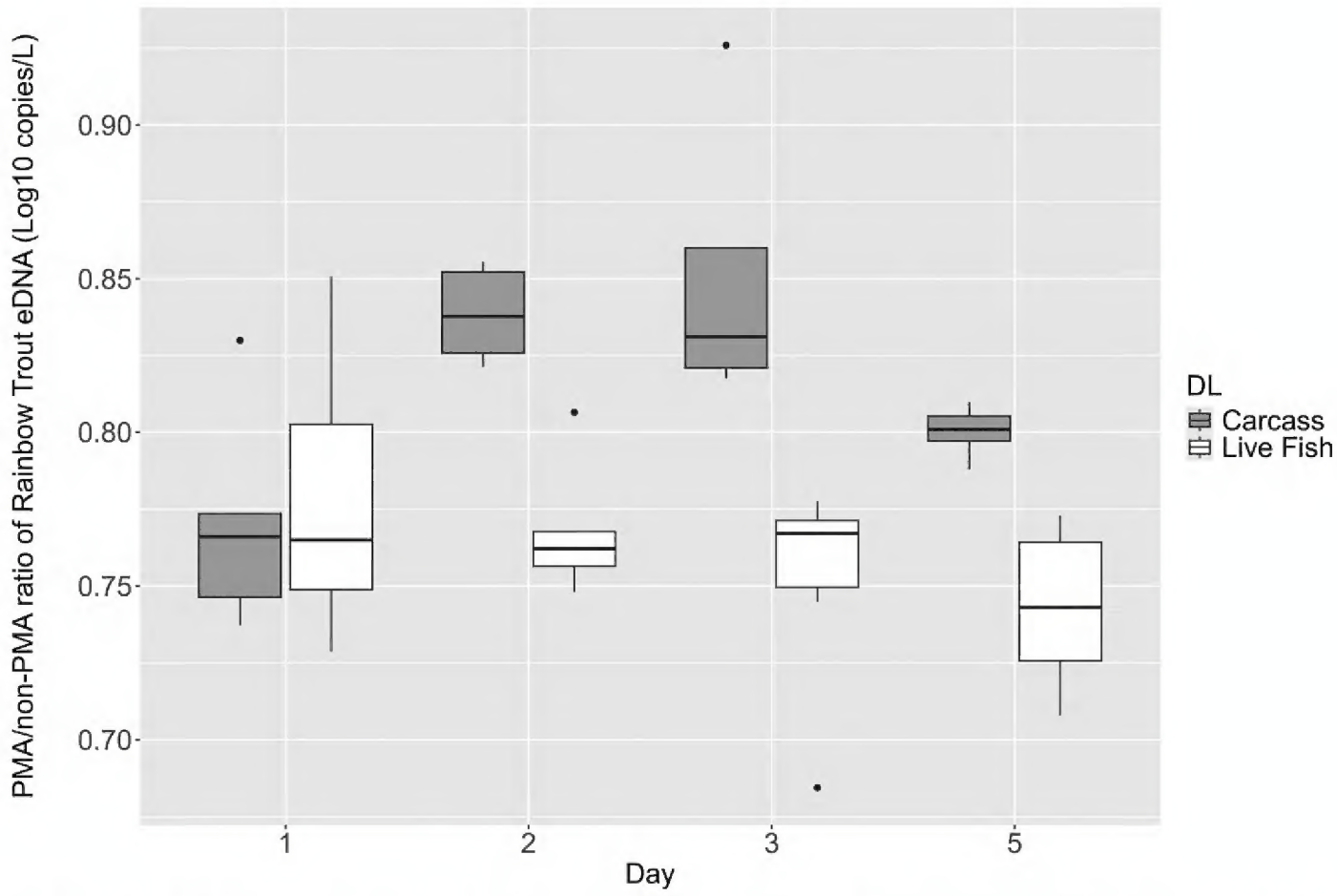

Figure 2. PMA/non-PMA ratio of log_{10}-transformed Rainbow Trout eDNA, based on the data shown in Fig. 1. In the boxplot, the solid central line represents the median. The lower and upper boundaries of the box indicate the 25th and 75th percentiles (the inter-quartile range: IQR), respectively. The upper and lower whiskers extend from the box to the highest and lowest values that are within 1.5 * IQR of the hinge. PMA stands for propidium monoazide.

the number of copies represented about 0.005% of the concentration found in Live Fish and Carcass blocks sampled on the same day and, therefore, contamination in the FNC Day3 was judged to be negligible and unlikely to affect the results. The other FNCs and NTCs (non-template controls) did not show any sign of contamination. All of the raw data obtained by real-time PCR are shown in Suppl. material 1 and in Ostberg et al. (2025).

Discussion

Our controlled laboratory experiments offer key insights into the dynamics of eDNA form and integrity, specifically inferring the contributions of live fish and carcasses to the release of intact cells and extracellular DNA. Carcass-derived DNA is known to generate false-positive detections in eDNA analysis (Merkes et al. 2014; Dunker et al. 2016), which has been highlighted as a concern to overcome, particularly in the context of invasive species eradication efforts (Kamoroff and Goldberg 2018). Moreover, when estimating biomass or abundance, carcasses have been confirmed as a significant contributor to the total eDNA concentration (Tillotson et al. 2018); this contribution must be accounted for to achieve accurate estimates. Despite the recognised importance of this issue, to our knowledge, the present study is the first to measure and compare eDNA shedding between live fish and carcasses while inferring the form of eDNA, which is important for understanding its dynamics (Jo et al. 2019).

In addition to the higher total eDNA shedding rate from carcasses, a major finding of this study was the higher proportion of PMA-resistant eDNA relative to total eDNA in the Carcass block compared to the Live Fish block. This

Table 3. Result of generalised linear mixed model (GLMM) analysis on the PMA/non-PMA ratio in Experiment 2. **(a)** Significance of each term in the model including interactions; **(b)** Output of multiple comparison testing between Live Fish and Carcass treatments for each Day; **(c)** Output of multiple comparison testing between Day in each of Live Fish and Carcass treatments. PMA and DL stand for propidium monoazide and dead or live, respectively.

(a)	Model term	df1	df2	F.ratio	p.value		
	Day	3	27	3.132	0.041932711		
	DL	1	9	30.504	0.000369024		
	Day:DL	3	27	5.544	0.004251873		
(b)	Day	estimate	SE	df	t.ratio	p.adjusted	
	1	-0.00734	0.0193	36	-0.379	1	
	2	0.0714	0.0193	36	3.691	0.0029	
	3	0.09899	0.0193	36	5.117	<.0001	
	5	0.0572	0.0193	36	2.957	0.0218	
(c)	DL	Day	estimate	SE	df	t.ratio	p.adjusted
	Carcass	Day1-Day2	-0.0679	0.0200	27	-3.396	0.0256
	Carcass	Day1-Day3	-0.0805	0.0200	27	-4.025	0.0050
	Carcass	Day1-Day5	-0.0296	0.0200	27	-1.479	1
	Carcass	Day2-Day3	-0.0126	0.0200	27	-0.629	1
	Carcass	Day2-Day5	0.0383	0.0200	27	1.917	0.791
	Carcass	Day3-Day5	0.0509	0.0200	27	2.546	0.2032
	Live Fish	Day1-Day2	0.0108	0.0183	27	0.594	1
	Live Fish	Day1-Day3	0.0259	0.0183	27	1.417	1
	Live Fish	Day1-Day5	0.0350	0.0183	27	1.915	0.793
	Live Fish	Day2-Day3	0.0150	0.0183	27	0.823	1
	Live Fish	Day2-Day5	0.0241	0.0183	27	1.322	1
	Live Fish	Day3-Day5	0.0091	0.0183	27	0.499	1

Degrees-of-freedom method: Kenward-Roger.
P value adjustment: Bonferroni method for 4 tests in the section b.
P value adjustment: Bonferroni method for 12 tests in the section c.

suggests that carcass-derived eDNA samples provided a higher concentration of intact cells, likely containing DNA with greater integrity. In research targeting microbes, it is desirable to use intact cells with high-integrity membranes to reduce the probability of contamination by exogenous nucleic acids, as this enables higher-quality analysis than analysing bulk DNA (Blainey 2013). Therefore, a treatment that sequesters potentially degraded DNA, including cells with compromised membranes and extracellular DNA, is necessary. The state and timing of cell release, particularly the high quantity of intact cells released shortly after organismal death, provides critical information on when and where to sample environmental waters to maximise the retrieval of high quality eDNA. Such samples can provide robust genotyping and population genomic analysis because high-quality, minimally degraded nucleic acids allow for more accurate inferences related to population structure. As indicated by Couton et al. (2023), the loss of individual information in current eDNA analyses is a significant limitation. Consequently, cell-by-cell analysis advances eDNA-based population genetic analyses that require information from multiple loci on single individuals, such as computing observed heterozygosity,

inbreeding coefficients and linkage disequilibrium. Researchers aiming to recover eDNA suitable for high-resolution genetic analyses as above may prioritise sites or time windows associated with recent, acute mortality events to enhance the yield of intact cells.

For eDNA analysis, it has been suggested that extracellular eDNA is highly susceptible to degradation driven by microbial activity (McKnight et al. 2024) and that relatively undegraded eDNA is likely intracellular (Jo et al. 2019). Targeting intact cells in this manner is crucial for performing higher-resolution genomic analyses in eDNA studies that focus on macroorganisms. However, even intracellular DNA can undergo degradation, likely due to intracellular nucleases (Bylemans et al. 2018). More specifically, Jo (2023) suggested that the presence of a cell membrane does not necessarily protect intracellular DNA from damage. Importantly, the term "intracellular DNA" used in previous eDNA studies on macroorganisms merely refers to the DNA captured in larger particle fractions through size fractionation, based on filter pore size during water sample filtration. In other words, to the best of our knowledge, no study in the field of eDNA analysis has yet elucidated the degree of DNA degradation exclusively within intact cells after explicitly confirming cell membrane integrity. When "cell-sized" particles are separated via filter fractionation and treated as membrane-bound DNA (Kirtane et al. 2023) or intracellular DNA (Bylemans et al. 2018; Jo 2023), it can be assumed that this fraction also contains membrane-compromised DNA. Therefore, calculating the actual degradation rate of intracellular DNA while enclosed within an intact membrane would require further detailed investigation.

One point worth noting is that the larger amount of total eDNA shed from carcasses can inflate abundance/biomass estimates, based on eDNA concentration. This could have implications for cases of mass mortality, which occur during the spawning migration of semelparous Pacific salmon in rivers of the Pacific Rim, for example. If live and dead fish shed eDNA at different rates and states, then dead fish could contribute disproportionate amounts of eDNA relative to live fish, potentially biasing the relationship between eDNA concentration and the abundance or biomass of target species (Tillotson et al. 2018), but note that this could be context specific (e.g. eDNA from crayfish carcasses was not detected in a stream mesocosm experiment; Curtis and Larson (2020)). To reduce the uncertainty in abundance/biomass estimates potentially caused by carcasses, it may be advisable to avoid sampling during mass mortality events.

Although we highlight the potential of PMA for eDNA analysis and application to genomics and population genetics, we stress that adopting PMA for this purpose would require optimisation of PMA protocols tailored for diverse eukaryotic eDNA targets, given the incomplete suppression of extracellular DNA that we observed in this study. The Rainbow Trout PCR assay that we used (Wilcox et al. 2015) had a 102 bp amplicon length and this short length could have contributed to the imperfect suppression of PCR amplification, based on other findings (Luo et al. 2010; Contreras et al. 2011; Hirohara et al. 2021). Contreras et al. (2011) assessed the importance of amplicon length (i.e. 140 bp versus 734 bp) on the effectiveness of PMA treatment. They found cycle delays in real-time PCR following PMA treatment for both amplicon lengths, but lower efficiencies for suppression by PMA for assays with short amplicons (140 bp), with 3.8 and 6.2 cycle delays reported for two bacterial species, *Vibrio anguillarum* and *Flavobacterium psychrophilum*, respectively. Conversely, they observed 13.3

and 17.6 cycle delays for the longest amplicons, concluding that increasing PCR amplicon length enhanced the effectiveness of PMA treatment. With respect to fish eDNA, the residual amount of DNA in a pasteurised treatment that was not suppressed by PMA was 12.0% of an untreated water sample using a PCR assay with 132 bp amplicon length (Hirohara et al. 2021). Based on previous observations that PMA efficiency increases with longer amplicon length (Contreras et al. 2011; Hirohara et al. 2021), the further reduction in PMA efficiency in our study relative to Hirohara et al. (2021) is consistent with using a shorter length of assay. Further studies examining how amplicon length affects PMA efficiency in eDNA are warranted.

Conclusion

In summary, with the use of a simple experimental study and assuming that PMA effectively eliminates free DNA, we demonstrated that fish carcasses were a robust and quantitatively greater source of intact cells compared with living fish, which shed more extracellular DNA and/or cells with disrupted membranes. Thus, our study fills a fundamental knowledge gap and provides a methodological stepping-stone towards the next generation of eDNA research. By informing sampling protocols and analytical strategies to leverage the availability of intact cells in eDNA samples, our work advances the application of eDNA approaches towards environmental genomics, including future developments in single-cell eDNA analysis. However, because the findings of this study are limited to Rainbow Trout under controlled conditions without additional confirmation of cell integrity using methods other than PMA, further research involving diverse species in both experimental and field environments would be needed to generalise the application of the PMA-based assessment of eDNA in intact cells.

Acknowledgements

We thank Gael Kurath and Doug McKinney (U.S. Geological Survey) for valuable assistance in designing our laboratory experiment and for providing study animals. We also thank Nobuhiro Mano (Nihon University, Japan) and Hisato Takeuchi (Ehime University, Japan) for providing information on viable PCR procedures. We appreciate thoughtful comments and very precise suggestions provided by the journal and internal reviewers. Any use of trade, firm or product names is for descriptive purposes only and does not imply endorsement by the U.S. Government.

Additional information

Conflict of interest

The authors have declared that no competing interests exist.

Ethical statement

The protocol we adopted in this study was approved by the Institutional Animal Care and Use Committee of the U.S. Geological Survey Western Fisheries Research Center (protocol# 2008-62).

Use of AI

The literature review of prior publications relevant to this study was conducted on Google Scholar (https://scholar.google.com/), but partly supported by literature search and intra-literature search functions of SciSpace (https://scispace.com) and NotebookLM (https://notebooklm.google.com). All cited publications were independently fact-checked by the authors.

Funding

This work was supported by the Joint Research Center for Science and Technology of Ryukoku University (2025), a grant for overseas research (kokugai-kenkyu) of Ryukoku University (2025–2026) to H. Y. and by the USGS Species Management Research Program. This research was also partly supported also by JSPS KAKENHI (23K05941) to H. Y.

Author contributions

H. Y. and T. H. conceived and designed the research. J. J. D. and C. O. O. significantly contributed to the refinement of the original research plan. All authors conducted the experiments. H.Y. wrote the first draft of the manuscript and all authors discussed the results and contributed to the development of the manuscript.

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Data availability

All data obtained in this study are available in Suppl. material 1 and also in Ostberg et al. (2025).

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Supplementary material 1

Additional information

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Link: https://doi.org/10.3897/mbmg.10.177451